Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/IL05/000028

International filing date: 09 January 2005 (09.01.2005)

Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 60/535,203
Filing date: 09 January 2004 (09.01.2004)

Date of receipt at the International Bureau: 31 January 2005 (31.01.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

World Intellectual Property Organization (WIPO) - Geneva, Switzerland
Organisation Mondiale de la Propriété Intellectuelle (OMPI) - Genève, Suisse

PCT/IL2005/000028
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APPLICATION NUMBER: *60/535,203*
FILING DATE: *January 09, 2004*

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This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. EV 325 705 548

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TITLE OF THE INVENTION (500 characters max)

USES OF BUTYRYLCHOLINESTERASE

Direct all correspondence to: CORRESPONDENCE ADDRESS

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ENCLOSED APPLICATION PARTS (check all that apply)

☒ Specification Number of Pages	28	☐ CD(s), Number	
☒ Drawing(s) Number of Sheets	2	☒ Other (specify)	Express Mail Certificate of Mailing dated January 9, 2004
☐ Application Data Sheet. See 37 CFR 1.76			

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Respectfully submitted, SIGNATURE _____ Date 01/09/04

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TELEPHONE 212 278-0400 Docket Number: 71837-PRO

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Dkt. 71837-PRO/JPW/KBC

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants : Hermona Soreq and Sophie Diamant

Provisional Application No.: Not Yet Known

Filed : Herewith

For : Uses of Butyrylcholinesterase

1185 Avenue of the Americas
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January 9, 2004

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Uses of Butyrylcholinesterase

Field of the Invention

The present invention relates to the field of amyloidoses and the formation of fibril structures from naturally non-fibrillated proteins. More specifically, this invention relates to amyloid plaque formation and the diseases that result from its accumulation. Yet more specifically, the present invention provides a novel use for the enzyme BChE, as a counter-acting factor for the process of fibrillation.

Background of the Invention

All publications mentioned throughout this application are fully incorporated herein by reference, including all references cited therein.

Butyrylcholinesterase (BChE, EC 3.1.1.8) is the primary circulating cholinesterase. It is abundant in serum and present at synapses and neuromuscular junctions, where it binds the same structural unit as the synaptic variant of acetylcholinesterase (AChE), AChE-S, with which it shares C-terminal sequence homology. It has a wide specificity for substrates and inhibitors, compared to the rather narrow specificity of AChE for its substrate.

BChE may serve the same biological function as AChE, which is hydrolysis of ACh at the end of each round of pre-synaptic secretion of ACh, binding of ACh to post-synaptic receptors and initiation of a post-synaptic action potential. At the neuromuscular junction, ACh released from the motoneuron terminal into the synaptic cleft travels toward the muscle post-synaptic membrane. There, it interacts with the nicotinic ACh receptor (AChR) to initiate an inward ion current that initiates a muscle action potential. ACh is subsequently hydrolyzed by AChE-S and BChE, both bound to the synaptic membrane by a structural subunit [Perrier, A. L. *et al.* (2002) *Neuron* 33: 275-285.]. Under

exposure to inhibitors, the muscle tissue produces, in addition to BChE and AChE-S mRNA transcripts, the normally rare AChE-R mRNA with its alternative 3' sequence. This transcript translates into soluble, secretory AChE-R monomers, which inhabit the synapse and hydrolyze ACh prior to its reaching AChR or after dissociating from it. Anti-ChEs block hydrolytic sites in AChE-S, AChE-R and BChE by a similar mechanism.

The proper folding of polypeptide chains is crucial for the normal function of proteins. However, various factors like stress or mutation may induce a cascade of yet incompletely understood processes leading to conformational changes or misfolding of a protein's structure. This usually involves the conversion from an α-helix configuration to a β-pleated sheet structure. These structural rearrangements, followed by nucleation, polymerization, aggregation and fibril formation, play a central role in the pathogenesis of most neurodegenerative diseases, such as Alzheimer's, Huntington's, Parkinson's and prion diseases and at least eight polyglutamine disorders [Kaytor, M. D. and Warren, S. T. (1999) *J. Biol. Chem.* 274(53): 37507-10], as well as various amyloidosis syndromes.

Amyloidosis is not a single disease entity but rather a diverse group of disease processes characterized by intra- or extracellular tissue deposits, in one or more organs, of fibril protein material which is generically termed amyloid. Amyloid is distinguished grossly by a starch-like staining reaction with iodine, microscopically by its extracellular distribution and tinctorial and optical properties when bound to Congo red or thioflavin T, or by its capacity to bind and induce fluorescence in bound thioflavin T, and by its protein fibril structure as shown by electron microscopy and X-ray crystallography.

The classification of amyloidosis (Table 1) is based upon the tissue distribution of amyloid deposits (local or systemic amyloidosis), the absence or presence of preexisting disease (primary or secondary amyloidosis), and the chemical type

of amyloid protein fibril. By convention, amyloid fibril types are designated by two letters: A for amyloid followed by a letter for the chemical type. There are two, chemically distinct, major types of amyloid protein fibrils designated AL and AA, respectively, and several minor types unrelated to AL or AA. AL (amyloid light chain) fibrils associated mainly with multiple myeloma are related to monoclonal immunoglobulin light chains synthesized by abnormal plasma cells. AA fibrils associated mainly with chronic inflammatory diseases are related to the non-immunoglobulin amyloid associated (AA) protein and its serum precursor (SAA), an acute phase reactant synthesized by liver cells.

Table 1: Classification of amyloidosis

Clinical Classification	Associated Condition	Amyloid Fibril Type	Precursor
Systemic Amyloidosis			
Primary or Secondary	Multiple myeloma	AL	Iglambda (or kappa chains)
Secondary	Chronic inflammatory disease, Rheumatoid arthritis, Tuberculosis, Skin and lung abscesses	AA	SAA
Secondary	Cancer Hodgkin's disease	AA	SAA
Secondary	Hemodialysis for CRF(*)	Beta2-m(**)	beta2-m
Primary	Heredofamilial amyloidosis, Familial Mediterranean Fever, Familial amyloid polyneuropathy	AA Transthyretin	SAA Transthyretin(#)
Localized Amyloidosis</TD< tr>			

Senile cardiac amyloidosis	Transthyretin	Transthyretin
Senile cerebral amyloidosis: Alzheimer's disease	Amyloid beta protein	Amyloid precursor protein (APP)
Endocrine tumors, Medullary carcinoma of thyroid	Procalcitonin	Calcitonin
Pancreatic islets β-cells	IAPP (Amylin)	IAPP (Amylin)

(*): chronic renal failure; (**): beta2-microglobulin is a normal serum protein and a component of MHC class I molecules; (#): transthyretin is a normal serum protein that transports thyroxin and retinol (vitamin A) and is deposited in a variant form.

An important pathological hallmark of Alzheimer's disease (AD) is the formation and progressive deposition of insoluble amyloid fibrils within the cerebral cortex. The key constituent of these amyloid deposits has been identified as a 39-43-amino acid long polypeptide, the β-amyloid peptide (Aβ). Once deposited as dense amyloid plaque cores, the peptide becomes highly resistant to further proteolysis and causes the dystrophy of the surrounding nerve cells [Knauer *et al.* (1992) *Proc. Natl. Acad. Sci. U.S.A.* 89(16): 7437-41; Nordstedt *et al.* (1994) *J. Biol. Chem.* 269(49): 30773-6].

In the past, it has been shown that AChE co-localizes with amyloid-β peptide (Aβ) deposits found in Alzheimer's patients brain [Inestrosa, N.C. *et al.* (1996a) *Mol. Psychiatry* 1(5): 359-61; Inestrosa, N. C. *et al.* (1996b) *Neuron* 16(4): 881-91]. Moreover, AChE not only co-localizes, but in fact promotes the aggregation of Aβ peptides and amyloid formation [Inestrosa , N. C. *et al.* (1996b) *id ibid.*]. The AChE-amyloid complexes present higher neurotoxicity than that of the Aβ aggregates alone [Alvarez *et al.* (1998) *J. Neurosci.* 18(9):3213-23]. In contrast,

despite the many structural and physicochemical properties that BChE shares with AChE, BChE does not promote amyloid formation [Inestrosa (1996b) *id ibid.*].

Thus, major research efforts have focused on uncovering the triggers and the effectors of these conformational conversions, aiming to develop workable therapeutic strategies to prevent amyloid plaque formation.

Interestingly, in all studies demonstrating AChE involvement and synergistic interaction with amyloid plaque formation, BChE was used as a neutral control, since it did not facilitate amyloid plaque formation [Inestrosa (1996b) *id ibid.*].

In the present invention, the inventors, who originally cloned the human BChE [US 5,215,909], set forth to investigate the involvement (if any) of BChE in fibrillation, the fibril deposit process. Unexpectedly, the inventors show that BChE counteracts AChE, slowing down the fibrillation process.

It may be understood that all fibril formation processes initiate by creation of an innoculum which facilitates fibril formation around it. Although in the different diseases related to fibril formation and deposition the proteins are different, the mechanism(s) supporting this event likely have a common basis and thus, based on the present results, the inventors propose that BChE serves to avoid such events in all different diseases.

Thus, it is an object of the present invention to provide novel uses for the enzyme Butyrylcholinesterase (BChE). More specifically, as it will become clear along the specification, the present inventors have found that BChE functions as a blocker of fibril formation, and thus may retard, prevent or slow down the progression of conditions that result from or are associated with fibril formation, like Alzheimer's disease, Parkinson's disease, Creutzfeldt-Jakob

disease, amyloidosis conditions (see Table 1), amongst others. Other uses and objects of the invention will become clear as the description proceeds:

Summary of the Invention

The present inventors have shown that the initiation of the process of fibril formation may be delayed and its rate decreased by BChE in 1:100 molar dose with the Aβ peptide.

Although the detailed mechanism by which BChE exerts this direct function is still unknown, the present invention provides, in a first aspect, the use of BChE as a direct blocker of fibril formation.

It is to be understood that by BChE it is meant the full-length protein, as well as any fragment, variant, analogue or derivative thereof which demonstrate the same activity, i.e., the capacity to block or protect against fibril formation.

BChE may be of human or mouse origin, preferably human.

In a second aspect, the present invention provides the use of BChE as a means to protect against fibril formation. In this aspect, BChE indirectly protects against fibril formation.

The present invention also provides the use of BChE as an indirect inhibitor of inflammation processes following injuries causing cholinergic imbalance, i.e. BChE could be used as an anti-inflammatory agent in these conditions. Similarly, BChE may be used to protect from the inflammatory consequences of organophosphate (OP) poisoning. Upon showing this capacity to block, inhibit or delay fibril formation, BChE may be used in the treatment of any one of diseases that are triggered by aberrant fibril formation and deposition. Examples of diseases of such origin are Alzheimer's disease (AD), Parkinson's

disease (PD), Creutzfeldt-Jakob disease (CJD), as well as any amyloidosis conditions.

In another aspect, the present invention provides the use of BChE as a therapeutic agent in the treatment of conditions that result from or are associated with fibril formation. Alternatively, BChE may be used as a prophylactic agent for patients at risk for conditions that result from or are associated with fibril formation.

The present invention provides the use of BChE to avoid hypersensitized responses to anti-cholinesterases, in particular in carriers of debilitating *BChE* mutations.

In a further aspect, the present invention also provides the use of BChE in the prevention of stress-induced damages in patients undergoing surgery.

The present invention is also concerned with providing methods that will prevent, slow down and/or retard fibril formation in subjects at risk, comprising administering a therapeutically effective amount of BChE to a subject in need.

Therefore, treatment with circulation-administered recombinant BChE may be developed as prophylactic as well as therapeutic to delay fibril formation.

The present invention provides a method of treatment of fibril formation-associated diseases, comprising administering a therapeutically effective amount of BChE to a subject in need, wherein said fibril formation-associated diseases are selected from the group consisting of AD, PD, CJD and amyloidosis conditions (see Table 1).

In one additional aspect, the present invention provides the use of BChE as a neutralizing agent for the effects of anti-cholinesterases, in particular for the treatment of patients suffering from cholinergic imbalance. For example, Myasthenia gravis (MG) patients suffer from cholinergic crisis, which involves massive overproduction of AChE. This condition cannot be treated with excess anti-cholinesterases (e.g. pyridostigmine), because patients cannot increase their production rate further. Thus this is likely a good indication for treatment with BChE. BChE shall scavange the excess inhibitors, hydrolyze the excess ACh inducing this crisis and retrieve cholinergic homeostasis.

In a further aspect, the present invention provides a method of reducing the levels of circulating ACh for subjects suffering from cholinergic imbalance, comprising administering a therapeutically effective amount of BChE to a subject in need.

In a yet further aspect, the present invention provides a method of treatment of conditions related to cholinergic imbalance, comprising administering a therapeutically effective amount of BChE to a subject in need, wherein said conditions are selected from the group consisting of head injury, viral infection, any condition increasing inflammatory risk.

Lastly, the present invention teaches a method of enhancing BChE expression by the use of transcriptional activators, comprising administering Vitamin D3 at 10^{-7} M to a subject in need, wherein said method is aimed at regulating the levels of circulating ACh, or circulating anti-cholinesterases.

The invention will be described in more details on hand of the following Figures.

Brief Description of the Figures

Figure 1: Effect of BuChE on amyloid formation.

a. Graph showing an increase with time of on-line Thioflavin T fluorescence incorporation into amyloid fibrils.
b. Histogram showing the rate of amyloid formation in the presence or absence of BChE.
c. Graph showing ThT fluorescence incorporation into amyloid fibrils in the presence of increasing concentrations of recombinant human AChE.
d. Graph showing decreasing ThT incorporation into amyloid fibrils in the presence of a constant dose of AChE and increasing doses of BChE.

Figure 2: Effect of AChE on amyloid formation.

This graph demonstrates the effect on yield of fibrils formed following the addition of increasing doses of AChE, as well as the decreasing lag period until fibril formation initiates.

Detailed Description of the Invention

In studying the process of fibril formation and deposition, responsible for the development of pathologies like Alzheimer's disease (AD), for example, the present inventors set up an *in vitro* system for fibril formation, and showed that the initiation of such process may be delayed and its rate decreased by BChE. The initiation of the process of fibril formation may be delayed and its rate decreased by BChE in 1:100 molar dose with the Aβ peptide.

Although the detailed mechanism by which BChE exerts this direct function is still unknown, the present invention provides, in a first aspect, the use of BChE as a direct blocker of fibril formation.

It is to be understood that by BChE it is meant the full-length protein, as well as any fragment, variant, analogue or derivative thereof which demonstrate the same activity, i.e., the capacity to block or protect against fibril formation.

A "fragment" is meant to refer to any peptide subset of the full-length BChE molecule. A "variant" of such molecule is meant to refer to a naturally occurring molecule substantially similar to either the entire molecule or a fragment thereof. An "analog" of a molecule can be without limitation a paralogous or orthologous molecule, e.g. a homologous molecule from the same species or from different species, respectively. The amino acid sequence of an analog or derivative may differ from said BChE of the present invention when at least one residue is deleted, inserted or substituted.

BChE may be of human or mouse origin, preferably human.

In a second aspect, the present invention provides the use of BChE as a means to protect against fibril formation. In this aspect, BChE indirectly protects against fibril formation.

As demonstrated in Example 1, BChE may be used to prevent AChE-induced formation of amyloid fibrils from APP, which occurs in AD patients.

Decreasing doses of ACh will induce inflammation [Tracey, K. J. (2002) *Nature* 420:853-859]. Therefore, therapeutic AChE treatment may increase the risk of inflammation, because the ACh levels will decrease. In contrast, higher levels of circulating BChE shall not induce inflammation, because BChE does not hydrolyze ACh as effectively as AChE does. But still, BChE has the ability to quench ACh. Thus, it may be concluded that BChE shall be able to attenuate the feedback auto-regulatory loop between ACh <-> AChE <->pro-inflammatory cytokines, which is triggered following injuries that induce cholinergic imbalance. Thus, the present invention also provides the use of

BChE as an indirect inhibitor of inflammation processes following injuries causing cholinergic imbalance, i.e. BChE could be used as an anti-inflammatory agent in these conditions. Similarly, BChE may be used to protect from the inflammatory consequences of organophosphate (OP) poisoning in humans as well as in animals, like domestic, pet or farm animals, and rodents (mice, rats), amongst others. Inflammation has been notably reported to increase the risk of fibril formation; therefore, BChE may also suppress fibril formation by preventing inflammation.

Upon showing this capacity to block, inhibit or delay fibril formation, BChE may be used in the treatment of any one of diseases that are triggered by aberrant fibril formation and deposition. Examples of diseases of such origin are AD, Parkinson's disease (PD), Creutzfeldt-Jakob disease (CJD), as well as any amyloidosis conditions.

Thus, in another aspect, the present invention provides the use of BChE as a therapeutic agent in the treatment of conditions that result from or are associated with fibril formation. Alternatively, BChE may be used as a prophylactic agent for patients at risk for conditions that result from or are associated with fibril formation.

There are over 100 natural and laboratory mutations of *BChE* [www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM]. One common BChE variant is called "atypical", which results from a point mutation responsible for the D70G substitution, and has a reported allele frequency up to 6% in certain populations [Ehrlich, G. *et al.* (1994) *Genomics* 22:288-295]. Carriers of this allele may suffer prolonged apnea following anesthesia, as well as sub-clinical cholinergic symptoms upon ingestion of glycoalkaloids present in the family *Solanaceae* (potatoes, tomatoes, aubergines), which are potent anti-ChE agents [Krasowski, M.D. (1997) *Can. J. Anaesth.* 44:525-534]. Thus, the inventors

suggest that BChE protects from dopaminergic damage under anti-cholinesterase, e.g. organophosphates damage.

The "dibucaine number" expresses the percent inhibition of BChE in the presence of a fixed concentration of dibucaine, and it is a convenient characterization of serum BChE variation [Kalow, W. and Genest, K. (1957) *Can. J. Biochem.* 35:339-346]. Carriers of debilitating BChE mutations have a dibucaine number of around 10%-25% of the normal value; these enzyme variants are deficient in their capacity to interact with anti-cholinesterase agents or hydrolyse acetylcholine [Lowenstein *et al.* (1995) *Nature Medicine*, 1: 1082-1085).

Thus, the present invention provides the use of BChE to avoid hypersensitized responses to anti-cholinesterases, in particular in carriers of debilitating *BChE* mutations.

In a further aspect, the present invention also provides the use of BChE in the prevention of stress-induced damages in patients undergoing surgery.

PON1 is an aryldialkylphosphatase, also known as paraoxonase, which hydrolyzes organophosphate (OP) substrates, which results in OP detoxification [Masson P. *et al.* (1998) *J. Physiol. Paris* 92:357-362]. PON1 is a glycoprotein associated with a subset of HDL molecules, which is produced and secreted in the liver, and exists in many tissues, particularly liver, kidney, small intestine and serum. This enzyme detoxifies OPs by hydrolyzing them, and prevents lipodoxidation of LDL and HDL [Mackness B. *et al.* (1998) *Gen. Pharmac, 31,* 329-336]. A decreased PON1 level obviously places an additional burden on the detoxifying function of BChE, especially under the stress of organophosphate exposure.

The *PON1* R allele has been reported to selectively increase the risk for coronary arterial disease (CAD) [Adkins S. *et al.* (1993) *Am. J Hum. Genet.* 52:598-608]. "Atypical" BChE was shown to increase this risk yet further [Nassar, B.A. *et al.* (2002) *Clin. Biochem.* 35(3):205-9]. Thus, the present invention also relates to the use of BChE to avoid cardiovascular disease in carriers of debilitating *PON1* mutations.

By debilitating *PON1* mutations it is understood any mutation (or allele) that result in lower PON1 serum activity. It is important to note that the PON1 status is defined as the combination of the genotype and the phenotype, the latter being affected by a high fat diet and exposure to xenobiotics – which reduce PON1 expression regardless of the phenotype.

The present invention is also concerned with providing methods that will prevent, slow down and/or retard fibril formation in subjects at risk, comprising administering a therapeutically effective amount of BChE to a subject in need.

The fact that BChE is capable of delaying the shift of an amorphous, unstructured polypeptide into a fibril network consisting of β-related sheets, as shown in Example 1, points at BChE as a putative natural protecting agent against such processes. Interestingly, PD involves parallel changes in the α-synuclein or Parkin proteins, and carriers of "atypical" BChE (with considerably lower BChE levels in their blood) are at increased risk for PD [co-pending Israel Patent Application No. 151955]. Circulating BChE levels in women are higher than in men, and indeed women are at lower risk of PD than men [Van den Eeden, S. K. *et al.* (2003) *Am. J. Epidemiol.* 157(11): 1015-22]. In comparison, Creutzfeldt-Jakob syndrome involves a parallel change in the prion protein, while cerebellar ataxia in a yet different protein. In essence, many if not all of the late age-onset neurodegenerative diseases involve structural changes in an initially unstructured neuronal protein which

suddenly shifts into insoluble, tightly woven networks, often neurotoxic. The β amyloid plaques of AD are another case of amyloidosis diseases, all of which follow the same principle, of amorphous peptides becoming insoluble precipitates that are toxic to the corresponding cells and tissues. The inventors hypothesize that the fibrillation process initiates in the circulation, where ample Aβ42 has been shown [Younkin S. G. *et al.* (1986) *Fed. Proc.* 45(13): 2982-8].

Therefore, treatment with circulation-administered recombinant BChE may be developed as prophylactic as well as therapeutic to delay fibril formation.

Hence, the present invention provides a method of treatment of fibril formation-associated diseases, comprising administering a therapeutically effective amount of BChE to a subject in need, wherein said fibril formation-associated diseases are selected from the group consisting of AD, PD, CJD and amyloidosis conditions (see Table 1).

Administration of BChE to a subject in need may be via oral, intravenous (i.v.) or intramuscular (i.m.). Preferably, administration is via i.v.

In US Patent Application USSN 10/337,142, by some of the present inventors, it was found that a double mutant mouse, bearing the mutant APP and overexpressing AChE, developed Aβ plaques considerably earlier than parallel mice with Aβ alone. This finding suggested that both the levels of Aβ42 and those of AChE are important for modulating the rate of the fibrillogenic process. The current findings demonstrate that BChE plays an additional, negative and important role in the process of plaque formation. It may be concluded that BChE functions as leverage in the cholinergic balance, helping to maintain healthy levels of circulating ACh and AChE.

In one additional aspect, the present invention provides the use of BChE as a neutralizing agent for the effects of anti-cholinesterases, in particular for the treatment of patients suffering from cholinergic imbalance. For example, Myasthenia gravis (MG) patients suffer from cholinergic crisis, which involves massive overproduction of AChE. This condition cannot be treated with excess anti-cholinesterases (e.g. pyridostigmine), because patients cannot increase their production rate further. Thus this is likely a good indication for treatment with BChE. BChE shall scavange the excess inhibitors, hydrolyze the excess ACh inducing this crisis and retrieve cholinergic homeostasis.

The BChE to be used in the invention may be natural, synthetic or recombinant. Recombinant BChE may be prepared by the method as described in WO 03/054182.

In a further aspect, the present invention provides a method of reducing the levels of circulating ACh for subjects suffering from cholinergic imbalance, comprising administering a therapeutically effective amount of BChE to a subject in need.

In a yet further aspect, the present invention provides a method of treatment of conditions related to cholinergic imbalance, comprising administering a therapeutically effective amount of BChE to a subject in need, wherein said conditions are selected from the group consisting of head injury, viral infection and any condition increasing inflammatory risk.

Lastly, the present invention teaches a method of enhancing BChE expression by the use of transcriptional activators, comprising administering Vitamin D3 at 10^{-7} M to a subject in need, wherein said method is aimed at regulating the levels of circulating ACh, as well as circulating anti-cholinesterases.

The present invention is defined by the claims, the contents of which are to be read as included within the disclosure of the specification.

Disclosed and described, it is to be understood that this invention is not limited to the particular examples, process steps, and materials disclosed herein as such process steps and materials may vary somewhat. It is also to be understood that the terminology used herein is used for the purpose of describing particular embodiments only and not intended to be limiting since the scope of the present invention will be limited only by the appended claims and equivalents thereof.

It must be noted that, as used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the content clearly dictates otherwise.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The following Examples are representative of techniques employed by the inventors in carrying out aspects of the present invention. It should be appreciated that while these techniques are exemplary of preferred embodiments for the practice of the invention, those of skill in the art, in light of the present disclosure, will recognize that numerous modifications can be made without departing from the spirit and intended scope of the invention.

Examples

Experimental Procedures

BChE biochemistry

Serum cholinesterase catalytic activity measurements are based on a spectrophotometric method adapted to a microtiter plate assay. Butyrylthiocholine (BTCh, Sigma) hydrolysis rates are measured following 20 min incubation with $5X10^{-5}M$ tetraisopropyl pyrophosphoramide (iso-OMPA, Sigma), a specific BChE inhibitor or $10^{-5}M$ 1,5-bis(4-allyldimethylammoniumphenyl) pentan-30one dibromide (BW284c51, Sigma, A9013), a specific AChE inhibitor. Addition of both inhibitors reduces hydrolysis to the rate of spontaneous hydrolysis measured in control reactions lacking enzyme or substrate, attesting to the specificity of these serum activities. Readings at 405 nm are repeated at 2-min intervals for 20 min. Non-enzymatic hydrolysis of substrate is subtracted from the total rate of hydrolysis. Enzyme activity is calculated using the molar extinction coefficient for 5-thio-2-nitrobenzoate [13,600 M^{-1} x cm^{-1}] [Ellman, G.L. *et al.* (1961) *Biochem. Pharmacol.* 7:88-95].

MPTP poisoning of mice

After its accidental discovery in the early 1980s, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) has been shown to induce Parkinsonism in monkeys, and Parkinson-like symptoms in mice, both at the behavioral and the anatomical level. Thus, MPTP has been used extensively as a model for Parkinson's disease in non-human primates and mice (Predborzski *et al.* (2000) *Restorative Neurology and Neuroscience* 16:135-142).

To prepare the MPTP solution, add 5 ml saline to one bottle of 10 mg MPTP, to obtain a 2 mg/ml solution. 0.01 ml of this MPTP solution is injected for each gram of mouse - e.g. a 20 g mouse receives 0.2 ml.

Telemetric follow-up of behavior

Battery operated biotelemetric transmitters (model VM-FH, Mini Mitter, Sun River, OR, USA) are implanted in the peritoneal cavity under ether anesthesia 12 days prior to the test. After implantation, mice are housed in separate cages with free access to food and water. Output is monitored by a receiver board (model RA-1010, Mini Mitter) placed under each animal's cage and fed into a peripheral processor (BCM 100) connected to a desktop computer. Locomotor activity after the dark/light shift is measured by detecting changes in signal strength as animals move about in their cages, so that the number of pulses generated by the transmitter is proportional to the distance the animal moves. The cumulative number of pulses generated over the noted periods is recorded [Yirmiya, R. *et al.* (1997) *Brain Res.* 749: 71-81]. Recording lasts 24 consecutive hours, starting at 9:30 am, with the light phase of a 12:12 h dark/light cycle beginning at 7:00 am. To initiate a day/night switch, the dark/light periods are reversed and recording starts 72 h after the switch and lasts 24 h. Recording proceeds for an additional 3 hours after injection of active agents.

ThT Fluorescence Measurement.

Aβ [1-40] was diluted from 2.23 mM stock solution in DMSO, into PBS (containing 0.02% Na-Azide and 0.88% glycerol), to the final concentration of 35μM. The incubation was performed at 30°C with shaking at 200 rpm, in the presence of 1μM ThioflavinT (ThT). The change in ThT fluorescence was followed with time (see representative experiment, Fig: 1a), using Tecan Spectro-Fluoromether with excitation wavelength of 450 nm and emission of 485 nm. Where indicated, the incubation solution contained BChE and/or AChE-S at the noted concentration ratio of Aβ to enzyme (Fig. 1b). The maximal rates of amyloid formation were derived from the time curves of the ThT fluorescence (Fig. 1a).

Example 1

The progressive deposition of amyloid β peptide (Aβ) in fibrillar form is a key feature in the development of the pathology in Alzheimer's disease. AChE is found associated with amyloid plaque deposits [Urlich, J. *et al.* (1990) *Acta Neuropathol. (Berl.)* 80(6):624-8]. In addition AChE, *in vitro*, directly promotes the assembly of Aβ peptide into amyloid fibrils [Alvarez *et al.* (1995) *Neurosci. Lett.* 201(1): 49-52; Inestrosa *et al.* (1996a) *id ibid.*; Inestrosa *et al.* (1996b) *id ibid*; Alvarez *et al.* (1998). *id ibid.*]. The fluorogenic incorporation of thioflavin T into Aβ fibrils essentially measures the shift from an amorphic, unstructured polypeptide into a tight network of β-pleated sheets which initiates the formation of the amyloid plaques.

In vitro Aβ fibrils form spontaneously, provided that the peptide is present at the certain critical concentration. At undisturbed conditions the fibrils are formed in the time span of days (2-7 days) but this process can be considerably accelerated by shaking the solution. The fibril formation can be followed by the measurements of: (1) turbidity of the solution, (2) staining with diazobenzidine sulfonate dye, Congo Red, or by (3) staining with bezothiazole dye, thioflavin T (ThT). The latter may be added at the end of the procedure, or, alternatively, at its beginning in which case it provides a real-time follow-up of fibril formation.

The inventors were able to follow the Aβ fibrils generation, *in vitro*, using all the above mentioned methods, with a preference for real-time ThT fluorescence measurements, as the most sensitive and reproducible method.

Figure 1 presents a typical experiment of measuring the kinetics of amyloid β sheets formation from $A\beta_{[1-40]}$ peptide (35 μM) in the absence or in the presence of recombinant hAChE-S (0.35 μM) or hBChE (0.35 μM), at 30°C, with shaking.

The presence of AChE decreases by half the lag time (from 200 min to 100 min) and increases the maximal rate of fibril formation (from 75 FU/min to 114

FU/min). In contrast, the presence of BChE increased the lag to 300 min and at 400 min the fluorescence ThT bound to amyloid fibrils was hardly significant.

The above described *in vitro* system enables the inventors to study the involvement of AChE variants, inhibitors and other AChE modifiers in the amyloid fibrils formation. This research shall contribute to the finding of novel therapeutic approaches for Alzheimer's disease, as well as all the conditions resulting from fibrils formation and deposition.

In sum, the present inventors show that human BChE, in a molar ratio of 1:100 to the Aβ peptide, is efficiently capable of slowing down the fibrillogenic process. Its presence retards the onset of fluorogenic increase and reduces the rate of that increase, once it was initiated. Moreover, when added to Aβ together with AChE, BChE is capable of delaying the onset and reducing the rate of fibril formation in a dose-dependent manner.

Example 2

In normal human serum, an average assay of 20 individuals yields 81±23 nmol butyrylthiocholine (BThCh) hydrolyzed/hr/μl serum (assayed at 2mM BThCh). Out of the total ACh binding sites in the human blood, BChE provides 75% or 50 nM [Lowenstein *et al.* (1995) *Mol. Cell Biol. Hum. Dis. Ser.* 5:307-49] and erythrocytes AChE – 25%, or 10nM [Ott *et al.* (1982) *FEBS Lett.* 138(2): 187-9]. However, the capacity of AChE to degrade ACh decreases above 3 mM (which is defined as "substrate inhibition"). In contrast, BChE's capacity to hydrolyze ACh increases under increased ACh concentration ("substrate activation"). Therefore, conditions of elevated ACh (i.e. acute stress injury or exposure to anticholinesterases) should best be treated with BChE, because under such conditions AChE's hydrolytic activity will be impaired but BChE's will be facilitated.

The present findings shed new light on yet more findings that associate the risk of neurodegenerative diseases with increases in the inflammatory load. Taking the inflammatory process as a protective mechanism, injury should induce an inflammatory response. This response requires increased production of pro-inflammatory cytokines (for example, by tissue macrophages). Intriguingly, the neurotransmitter which controls this process is acetylcholine (ACh) [Bernik, T.R. *et al.* (2002) *J. Exp. Med.* 195(6):781-8]. In both tissues and circulation, ACh levels are controlled by AChE [Soreq, H. and Seidman, S. (2001) *Nature Neurosci. Rev.* 2:294-302]. Therefore, increased AChE can initiate inflammatory reactions because it reduces ACh levels and increases production of cytokines. However, while inflammatory reactions are apparently useful in the short range, they carry a significant long-range risk of neurodegenerative disease. For example, head injury induces the largest non-inherited risk of AD [Shohami, E. *et al.* (2000) *J. Mol. Med.* 78:228-236]. Therefore both therapeutic uses of recombinant AChE and treating patients with anti-cholinesterases, which induces AChE overproduction as a feedback response, carry an inherent risk of increasing the inflammatory load in treated patients.

Unlike AChE, BChE does not entail a risk of increasing the inflammatory load, since it is not part of the feedback auto-regulatory loop of injury-cytokine release-cholinergic imbalance.

In addition, BChE is soluble and thus accessible to circulating AChE. Moreover, the substrate of preference is different. Over the range of substrate concentrations 0.1 to 25 mM, the ratio of hydrolysis of AThCh relative to BThCh differ as follows (Table 2):

Table 2: Rate of hydrolysis of AThCh/BThCh*

Substrate (mM):	0.1	25.0
hSerum BChE	0.45	0.20
hRBC AChE	120	7.2

*Average of 3 experiments.

Thus, under normal condition, and assuming low ACh levels and negligible soluble AChE levels in the circulation, the total ACh hydrolyzing capacity will be divided as follows:

75% - BChE

25% - AChE

3:1 ratio

Based on the AThCh hydrolysis ratio differences of AChE:BChE [245-fold at 0.1 mM]; BChE's capacity of ACh hydrolysis in the circulation will be ca. 80-fold lower than that of AChE under normal circumstances. However, increased ACh levels (up to 10 mM, as is the concentration in synapses) will improve BChE's capacity to hydrolyze ACh (as in 25 mM, the difference is 36-fold, or only 12-fold lower than AChE). Nevertheless, BChE administration shall not increase the inflammatory load, because even at these higher ACh concentrations BChE will only constitute 10% of the circulation capacity to hydrolyze ACh. The progressive age-dependent increase in inflammatory diseases should hence be attributed to the increase in circulation AChE, not to BChE. Mice injected with paraoxon, the OP metabolite of parathion, show reduced locomotion and decreased body temperature. Injected hrBChE (human recombinant BChE) will limit this response and reduce production of pro-inflammatory cytokines.

Example 3

BChE reduces caspase 9 activation in OP-exposed mice

In order to measure caspase activity, the following assay is used in which caspase activity leads to cleavage of a substrate, resulting in the release of pNA, which can be detected in a microplate reader at 405 nm. The assay comprises the following steps:

1. Induce cells to undergo apoptosis.
2. Remove media, wash cells, and scrape cells into PBS.
3. Pellet cells gently and resuspend in cell lysis buffer (10 mM HEPES, 2 mM EDTA , 0.1 % CHAPS or NP40, 5 mM DTT, 1 mM PMSF, 10 μg/ml pepstatin A, 20 μg/ml leupeptin, 10 μg/ml aprotinin).
4. Incubate on ice for 10-15 minutes.
5. Centrifuge at 10 000g for 1 minute at 4°C.
6. Transfer supernatant to a new tube and keep on ice.
7. Measure protein content of the supernatant.
8. In a microplate reader add: 178 μl reaction buffer (100 mM HEPES, 20%v/v glycerol, 0.5 mM EDTA, 5 mM DTT) up to 20 μl cell lysate (containing 100-200 mg total protein) and 2 μl caspase substrate. Adjust volume to 200 ml with cell lysis buffer if required.
9. Incubate at 37°C and measure the absorbance at 405 nm every 30 minutes.

It is necessary to use the following controls: i) uninduced cells ii) cell lysate only (with no caspase substrate) iii) substrate only (with no cell lysate). The readings from controls ii and iii show that show the background absorbance and can be subtracted from the experimental readings. The first control gives a value for the endogenous caspase activity in the cells.

Example 4

Carriers of higher BChE levels are less susceptible for cytokine elevation following surgery

It is well known that stress influences many immune functions, including the production and secretion of cytokines. Following exposure to various stressors, there is an increase in peripheral IL-6, as well as IL-1β and TNFα, accompanied by decrease in IL-2, in both humans and experimental animals.

In co-pending Israel Patent Application No. 158600, a study was designed to examine the role of cytokines in mediating the affective and cognitive effects of stress, twenty generally healthy volunteers were administered with a comprehensive neuropsychological test battery, assessing emotional and cognitive parameters, before and after a minor surgery. In addition, blood samples were collected in each session, and serum levels of cytokines (IL-1β, IL-6) were measured. The results of this study showed that in the morning of the surgery day, there was a significant increase in the levels of both anxiety and depression. No change was found in the levels of fatigue and pain. In the morning following surgery there was further increase in depression, but not in anxiety, alongside a significant increase in pain and fatigue.

With regards to the cognitive parameters, in the morning of the surgery day tests showed a significant decline in performance of the word list recall task (HVLT). In the morning following surgery, an additional decline was found in the word list recall as well as in the performance of a visual memory task involving a complex figure reconstruction. Most importantly, in the morning of the surgery day, there was a significant correlation between increased levels of IL-1β and the elevation in depressed mood. In the morning following the surgery, there were significant correlations between increased IL-1 levels and impaired immediate and delayed Logical memory (story recall test). Significant correlations were obtained between increased IL-6 levels and improved delayed recall in the Word List Recall test, as well as improved

immediate and delayed Complex Figure recall test. There was a significant increase in IL-6.

Administration of BChE to patients undergoing surgery shall prevent or diminish this pro-inflammatory response and the secretion of pro-inflammatory cytokines, particularly IL-1 and IL-6.

Example 5

BChE limits anxiety phenotypes

Individuals with low or high serum BChE activities who are under environmental OP exposure will be tested psychologically for anxiety.

A report by some of the inventors [Sklan et al. *Proc. Natl. Acad. Sci. U.S.A.* (submitted)] showed correlations between serum biochemical markers and trait anxiety. Testing individuals who presented the top 20% trait anxiety scores against the rest of the population using a generalized linear model (with a logistic link function) showed a significant effect of both the genotyped polymorphisms ($p<0.013$) and the serum activity levels of AChE, BChE and PON ($p<0.022$) on the trait anxiety score. In a regression analysis testing for factors contributing to the trait anxiety scores, there was a clear effect of all the polymorphisms genotyped together with gender, age and ethnic origin on PON serum activities ($p<5.4x10^{-7}$). A smaller, but significant effect was observed on AChE ($p<0.03$) and BChE activities ($p<0.04$). Thus, the inherited parameters which contribute significantly to the measured enzyme activities further predict a considerable fraction of the quantified trait anxiety scores with significant power.

In view of these results, it may be predicted that treatment with BChE may limit the anxiety phenotype in individuals with naturally low BChE activity.

Claims:

1. Use of BChE as a blocker of fibril formation.

2. Use of BChE for protecting against fibril formation.

3. Use of BChE as an anti-inflammatory agent associate with any one of injuries causing cholinergic imbalance and inflammatory processes resulting from organophosphate poisoning.

4. Use of BChE to prevent AChE-induced formation of amyloid fibrils in Alzheimer's disease (AD) patients.

5. Use of BChE to avoid cardiovascular disease in carriers of debilitating *PON1* mutations.

6. Use of BChE to avoid hypersensitized response to anti-cholinesterases in carriers of debilitating BChE mutations.

7. Use of BChE in the prevention of stress-induced damages in patients undergoing surgery.

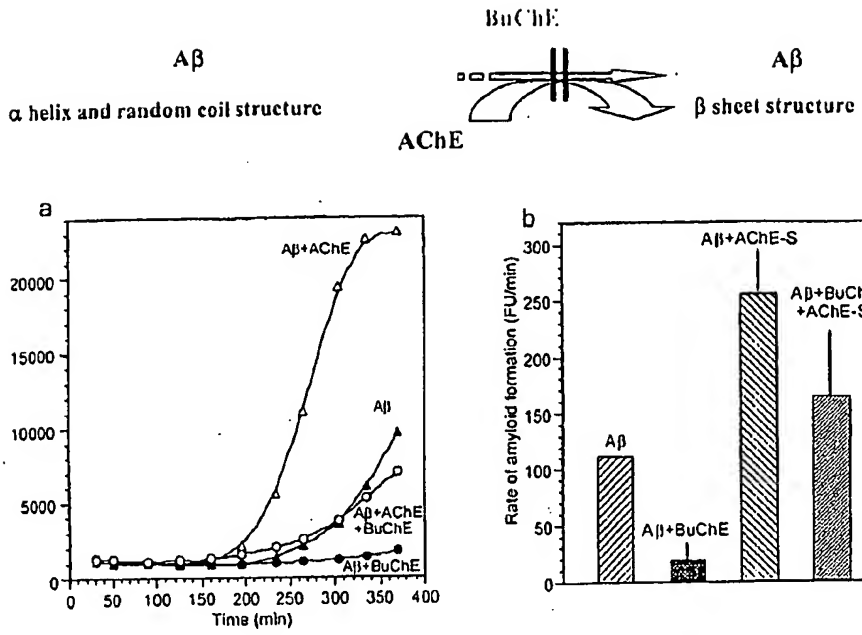
8. The use of any one of claims 1 or 2 in the treatment of any one of AD, Parkinson's disease (PD), Creutzfeldt-Jakob disease (CJD), and any amyloidosis condition.

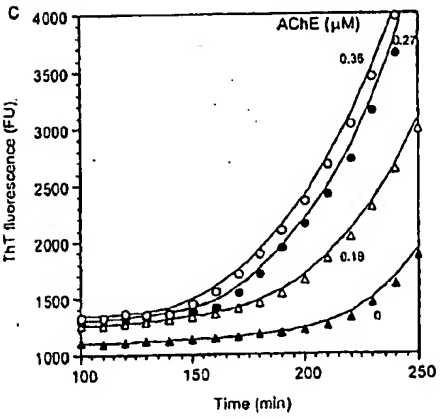
9. Use of BChE as a therapeutic agent in the treatment of conditions that result from or are associated with fibril formation.

10. Use of BChE as a prophylactic agent for patients at risk for conditions that result from or are associated with fibril formation.

subject in need, wherein said conditions are selected from the group consisting of head injury, viral infections, and any condition increasing inflammatory risk.

20. A method of enhancing BChE expression, comprising administering Vitamin D3 at 10^{-7} M to a subject in need, wherein said method is aimed at regulating the levels of circulating ACh, or circulating anti-cholinesterases.





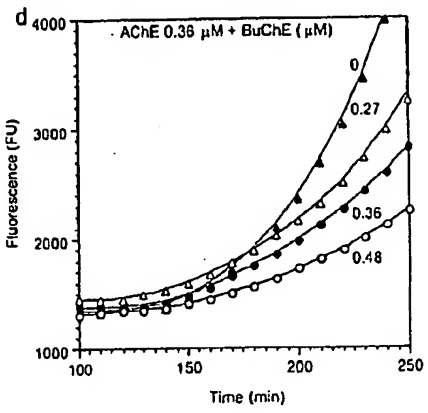


Fig. 1

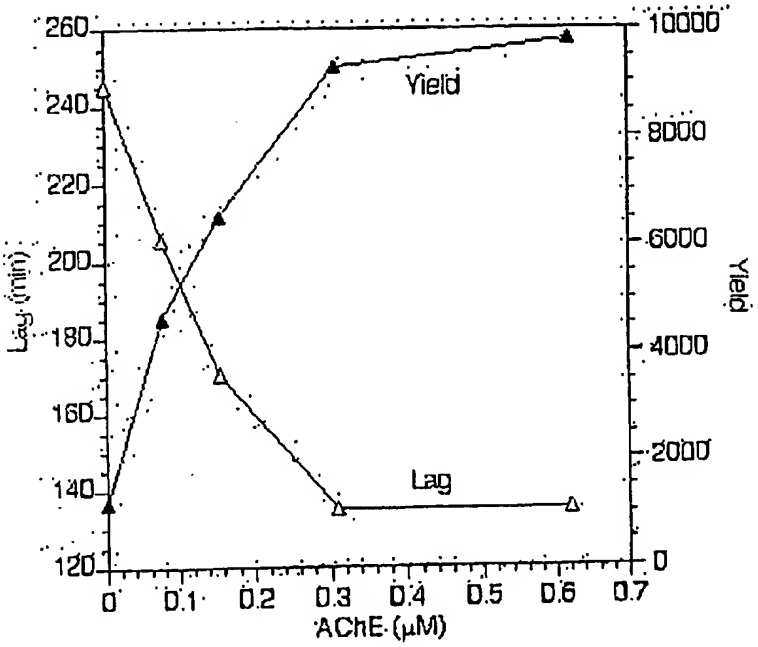


Fig. 2